4347

Isolation of Brassins by Extraction of Rape (*Brassica napus* L.) Pollen

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Rape pollen was used as a source for the production of new natural plant growth-promoting substances, brassins, which contain mostly mixtures of plant lipids. A pilot-scale extraction method was devised to isolate brassins in relatively large quantities. This method utilizes the extraction of rape pollen with 2-propanol followed by solvent partition and a two-step column chromatographic procedure to give biologically active brassins. Similar extraction procedures are suggested for the isolation of natural growth-regulating substances present in minute amounts from other plant sources.

Introduction

Since we reported (Mitchell et al., 1970; Mandava, 1971; Mandava and Mitchell, 1971a) that brassins exhibited unusual growth effects, our attention has been directed to the chemical identification of the biologically active constituent(s). This group containing lipoidal substances has been isolated from rape (*Brassica napus* L.) pollen and has caused growth effects that have never been noted with the known plant hormones (Worley and Mitchell, 1971; Mitchell et al., 1971a; Worley and Krizek, 1972; Mitchell and Gregory, 1972). The active constituents are present in minute quantities; several attempts were made previously to identify them by microanalytical techniques such as GC–MS, but with little success (Mandava and Mitchell, 1972; Grove et al., 1978). Earlier we reported (Mandava et al., 1973) a convenient method for obtaining brassins on a laboratory scale and this method enabled us to produce enough brassins for a series of greenhouse and field experiments. Larger quantities were required for detailed chemical identification. Therefore, we devised a pilot plant size procedure for the extraction of pollen followed by purification of the crude extract. In this paper, we report an isolation method that is useful for the production of brassins and also can be adapted for plant growth-regulating substances from other natural sources (Mandava and Mitchell, 1971b; Mitchell et al., 1971b).

Experimental Section

Materials used. Rape pollen collected by honeybees in rape fields was obtained from Canada. All solvents used for extraction and isolations were reagent grade. Silica gel 60 (70–230 mesh) and preparative silica gel (thick layer) plates were obtained from EM Laboratories, Inc., Elmsford, N.Y.

Apparatus. A Coleman Junior spectrophotometer (Model 6A) (Coleman Instruments, Maywood, Ill.) was used for monitoring column chromatographic fractions. All evaporations were carried out either on a laboratory concentrator (GCA/Precision Scientific Co., Chicago, Ill.) or a large Rotavapor (Büchi/Brinkmann Rotavapor R-10, Brinkman Instruments, Inc., Westbury, N.Y.).

Pollen Extraction. (1) Laboratory Method. Experiments were carried out to determine suitable pilot plant processing conditions. Rape pollen was washed for 30 min with deionized water (4 mL of water/1 g of pollen) and filtered; the process was repeated seven times. The filter cake was dried to less than 1% water and then extracted for 1 h with 2-propanol (3 mL of solvent/1 g of pollen). Extractions (6) were carried out until the biological activity of the last extract showed minimal (or marginal) activity on the basis of the amount of extractable active material per unit volume of 2-propanol solution.

(2) Pilot Plant Extraction. The pilot plant extraction procedure is shown in Figures 1 and 2. Rape pollen (45.5 kg) was extracted with deionized water (20 μmho) in a 227-L scraped wall kettle, Hamilton style A, double motion scraper agitator (Hamilton Copper and Brass Works Co., Hamilton, Ohio). The slurry was filtered in a Sparkler filter, size 18D10 (Sparkler Manufacturing Co., Conroe, Texas), using A-7 filter paper. The filter cake was continuously washed with deionized water until the filtrate was clear and virtually colorless. The washed pollen (filter cake) was freeze-dried in an F. J. Stokes Corp. (Philadelphia, Pa.) Model 338P dryer. The freeze-dried pollen was then extracted with about 114 L of 2-propanol in the Hamilton kettle and the slurry pumped to the Sparkler

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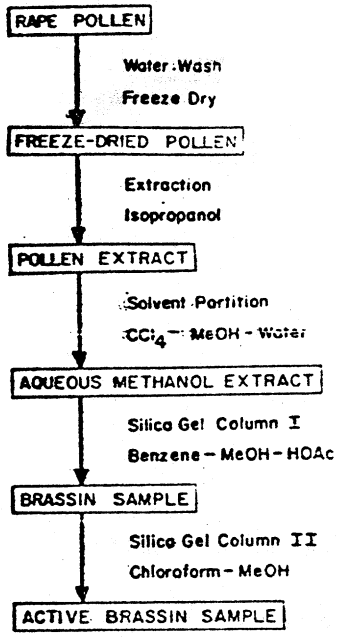

Figure 1. Flow diagram of the isolation of brassins from rape pollen.

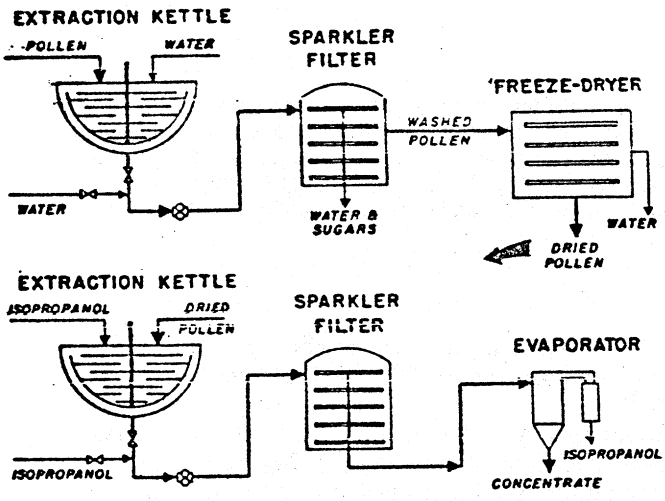

Figure 2. Diagram showing the pilot plant extraction process.

filter. For each subsequent extraction, the filter was filled with 2-propanol, soaked, then drained (about 114 L per extraction). The pollen filter cake was washed in this manner in the filter six times (one extraction and six washes were considered seven extractions). For the eighth extraction, the filter cake (pollen) was recycled to the Hamilton kettle and the extraction was repeated. After filtration, the pollen was washed twice and then recycled to the Hamilton kettle for one more extraction. Thus, each batch was leached with 2-propanol 11 times (three in the kettle and eight in the filter). Bioassay results indicated that extraction of active material was essentially complete by the eighth extraction (two in the kettle).

The individual filtrates from all extractions and washings were evaporated on a Precision Scientific, 3-L (D-1) laboratory glass evaporator. The temperature was maintained below 40 °C (above 40 °C biological activity is destroyed). The total yield of 2-propanol extract from 227 kg of pollen (five 45.5-kg batches) was 38 L of concentrate. Suspended solids were removed from the concentrate in a large centrifuge (International Equipment Co., Mass., Size 2, Model V). The liquid was further concentrated in vacuo to give 16 kg of a brown syrupy concentrate.

Solvent Partition of the Pollen Extract. The extract (16 kg) was partitioned between methanol and carbon

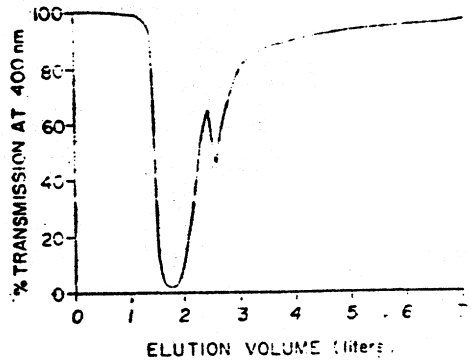

Figure 3. Percent transmittance profile at 400 nm of the eluates from silica gel column chromatography of pollen extract.

tetrachloride in several batch lots. The general procedure for batch lots consisted of dissolving the extract (2 kg) in carbon tetrachloride (1000 mL) and placing the resulting clear solution in a separatory funnel. Water (100 mL) was added and the mixture was thoroughly shaken; on standing, a two-phase system was developed. A set of five separatory funnels was arranged in series and a mixture of carbon tetrachloride (400 mL), methanol (200 mL) and water (26 mL) was placed in each separatory funnel. The lower phase in the separatory funnel that contained the carbon tetrachloride solution was transferred to the first funnel in the set of five funnels. After shaking and allowing the layers to separate, the lower phase was transferred to the next funnel and the procedure was repeated continuously. After six transfers, the aqueous methanol layers which contained essentially all the biological activity were combined and the solvent was evaporated to give a dark brown gum (90 g). Each 200-g batch of extract took about 2 h to complete so that 800–1000 g of the extract was partitioned in 1 day by a single person. The solvent from the combined batches was evaporated. Thus the 2-propanol extract (16 kg from 227 kg of pollen) was reduced to about 7.5 kg by this method.

Initial Column Chromatography. Chromatographic columns (85 × 9 cm o.d.) were packed with silica gel (1500 g) in benzene–methanol–acetic acid (90:16:8) to give a bed length of about 50 cm and then washed with the same solvent mixture (700 mL). The aqueous methanol material (100 g for each column) was mixed with silica gel (1 g of material/2 g of silica gel) using 2-propanol which was then removed at 40 °C under vacuum to give a brown powder. This powder was applied to the columns which were eluted with benzene–methanol–acetic acid (90:16:8). The eluates from the column were monitored using a spectrophotometer by measuring percent transmittance at 400 nm. The biological activity that was correlated with percent transmittance (Figure 3) appeared after the first 3 L of solvent eluted from each column. By the time the next 3 L had been collected, the percent transmittance had nearly reached a maximum (96–98%). Individual fractions (1 L) were combined after assaying for biological activity. In this manner, each column required about 7 L of the solvent mixture to yield 5–7 g of material containing activity. Two to four columns were run simultaneously, the active fractions were combined, and the solvent was evaporated. About 450 g of the active material (from 181 kg of pollen) was obtained.

Second Column Chromatography. The active material from the first silica gel column was further purified by a second silica column (85 × 9 cm o.d.) packed with 1500 g of silica gel in chloroform. The material (60 g) was applied and successively eluted with the increasing concentrations of methanol in chloroform. All the biological

ty was eluted from the column when the methanol entration was raised to about 10–20% and the total ive brassin sample of ~200 g represented the 181 kg rape pollen.

Bioassay. All the samples, from crude pollen extracts o purified brassin samples, were tested on a bean second nternode, by a bioassay procedure which has been described (Mitchell and Livingston, 1968). All the active amples showed typical brassin activity that included evere swelling and curvature and splitting of the treated nternode.

Results and Discussion

Earlier we reported a convenient method for obtaining brassins on a small scale. Using this method, we were able o produce milligram quantities of brassins sufficient for imited biological and field experiments. Since the active onstituents are estimated to be present below 0.1 ppm, ve required a pilot plant extraction method as reported n this paper. The present method consists essentially of ive steps: (i) extraction of the pollen with water, (ii) reeze-drying the washed pollen, (iii) extraction of the dried ollen with 2-propanol, (iv) solvent partitioning of the -propanol extract, and (v) purification of the active queous methanol fraction by two-step column chromaography.

We previously recommended the use of ether as an xtraction solvent for the pollen since an ether extract ontained less impurities. Although it served our purpose t that time, we had to replace ether in the present method or the following reasons: (1) ether did not extract all the iologically active material; (2) ether does not penetrate vater-washed pollen; and (3) there is a hazardous nature f ether in the pilot plant extraction. Among the solvents valuated (several hydrocarbons, alcohols, ethers, and sters), 2-propanol appeared to be the best compromise or the extraction purposes. We found that preliminary vashing of the pollen with water removed many simple ugars which do not contain biological activity. This was ollowed by a freeze-drying step which aided in breaking p the pollen cake. Repeated extractions of the freeze-ried pollen with 2-propanol gave material containing iological activity. In our experience, we did not extract ll the activity from the pollen, but the activity (based on he amount of extract) remaining in the pollen was reduced onsiderably after about six extractions. Since the nature f the active compounds is unknown, we avoided drastic onditions such as Soxhlet-type extraction at the boiling emperature of 2-propanol (maximum temperature used vas 40 °C). Other hydroxylic solvents such as methanol nd ethanol were also suitable for the extraction, but we hose 2-propanol because of its low volatility, low flamnability, and ease of operation under pilot plant conditions. Another reason we chose 2-propanol is that the xtracted material after solvent partitioning with carbon etrachloride–methanol was readily soluble in 2-propanol. Otherwise, considerable difficulty was encountered in edissolving the residue after evaporation.

Before the large extractions were undertaken, some imulation experiments were carried out to establish uitable conditions for the pilot plant. Rape pollen was oaked in deionized water with shaking for 30 min and iltered. A ratio of 4 mL of water to 1 g of pollen was found uitable for the washing and the pollen was washed with vater seven times to remove most of the soluble free ugars. Drying the water-washed pollen presented roblems since the wet pollen was sticky and removal of vater by vacuum-drying sometimes resulted in rancid naterials. We found that freeze-drying the washed pollen was effective and also tended to break up the pellets as determined by microscopic examination. Individual freeze-drying was performed on the 45.5-kg batches of pollen following washing with water. Solvent extraction of the freeze-dried pollen was performed first in an agitated kettle and then the material was pumped into the Sparkler filter. The filter cake was washed (at least six times) repeatedly with fresh solvent. The cake was then transferred to the kettle and the extraction procedure was repeated. Such repeated operations gave several hundred liters of filtrate which were concentrated on an evaporator. To determine how many times the extraction process needed to be repeated, aliquots from concentrates of the individual extractions and washings were purified by TLC and the R_f values were compared with that of a standard brassin sample. Biological activity of the samples before and after TLC clean-up was monitored. We used two criteria for terminating the extraction: (a) amount of extractable material and its relative activity in each extraction, and (b) comparative biological activity both by the bean second internode assay and by a seed germination assay on TLC plates (H. Kenney, unpublished).

After having successfully performed the pilot plant extraction of the rape pollen, the next step was purification. In our previous paper (Mandava et al., 1973), we used a two-step purification, viz., silica gel column chromatography followed by TLC. Subsequently, the TLC step was also modified by using a second silica gel column (50:50 isopropyl ether–EtOH) in place of TLC to produce a comparable material. In the present method, we have introduced a solvent partitioning step for three reasons: (1) to eliminate the coagulants from the 2-propanol pilot-plant extract which clog the columns, (2) to allow application of extracts on the columns since residual 2-propanol after evaporation under vacuum did not allow direct application (complete evaporation of the samples makes transfer difficult and very time consuming), and (3) to eliminate the use of several columns for reduction of inert and growth-inhibiting substances in the extract. We investigated several solvent partitioning systems which included the following: (A) carbon tetrachloride–methanol–water (2:1:0.1), (B) hexane–methanol–water (50:45:5), (C) hexane–2-propanol–water (50:40:10), and (D) ethyl acetate–methanol–water (50:40:10). We found solvent systems A and B best, but we had to choose system A because we encountered problems with emulsions with system B, especially when large volumes of solvents were handled. Solvent system A presented no problems and the biological activity remained in the aqueous methanol phase.

Large silica gel columns that handle 100 g of the pollen extract at a time were employed and could be used repeatedly (6–10 times). Elution with benzene–methanol–acetic acid gave several liters of the eluate. Earlier we reported the use of color standards and an RI monitor to detect biological activity in the column eluates (Mandava et al., 1973). Later we found that recording the percent transmittance at 400 nm during elution was more sensitive and a reliable method. In general, the biological activity did not appear until percent transmittance dropped to a minimum (Figure 3). As the percent transmittance began to increase, the biological activity was found in the eluates and the activity eluted before the plateau was reached. Although there were slight variations in the percent transmittance profiles among different batches of pollen, there were advantages of this monitoring technique: (1) in the absence of bioassay, we were able to rely on this physical method; (2) it is many times more sensitive as a

selection method than the previously adapted IR and color comparison methods: (3) it can be utilized for any size column; and (4) repeated use of the same column (silica gel) will not greatly alter the transmittance profile.

The individual fractions of eluate that showed biological activity and light absorption in the 60–90% transmittance range were combined. The combined sample was purified on a second silica gel column and the biological activity was eluted with 10–20% methanol in chloroform. Using the general outline (Figure 1), 181 kg of pollen resulted in about 200 g of biologically active material which showed typical brassin response.

Plant hormones are present in very minute amounts in most plant tissue (Mandava, 1973). Recently, the presence of these substances has been estimated by several sophisticated and highly sensitive techniques such as mass spectrometry and fluorimetry. The levels of indole-3-acetic acid (Rivier and Pilet, 1974) and abscisic acid (Rivier et al., 1977) were about 2 and 60 ppm, respectively. The active component(s) for brassins is estimated to be below 0.1 ppm. Progress in the identification of many plant hormones has been hindered due to the lack of sufficient amounts of material. Laboratory extractions yield small quantities and are very time consuming. It must be pointed out that no attempt has been made thus far to extract the plant tissue for the isolation of plant growth-regulating substances on a pilot plant scale, although, in a few instances, large-scale laboratory extractions to isolate milligram quantities have been reported (Murofushi et al., 1966; Ohkuma et al., 1963). To our knowledge, this represents the first report on the pilot plant extraction of a plant tissue for speedy and efficient

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Thiol Synthesis from Carbonyl Compounds

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A new method for the preparation of thiols from corresponding carbonyl compounds is described. It consists of the catalytic action of H_2S on a carbonyl compound in the presence of a thiol acting as a reducing agent. The conditions of temperature and pressure are mild. K_2O–alumina with about 5% of K_2O is the most active, selective, and stable catalyst. Methanethiol is preferred as a reducing thiol because it is economical and the easiest to eliminate from the thiol produced. This method gives all primary and secondary thiols with an excellent yield.

Introduction

The synthesis of thiols can be achieved by various methods using different raw materials. However, there is no method which is both selective and easily applied for the preparation of all primary and secondary thiols.

The reaction of H_2S with alkenes constitutes the simplest method (Bapseres, 1963), but two thiols can be formed from asymmetrical alkenes. The reaction of metallic sulfhydrates with alkyl halides represents another means of synthesis. Yet it is not easy to obtain pure secondary halides. Indeed their isomerization is rapid and their compounds are difficult to separate from their isomers (Bunnett and Leck, 1973). The same problems are encountered in the reaction of an alkyl halide with thiourea (Cossar et al., 1962).

Primary and secondary thiols are also obtained by the reductive thiolation of aldehydes or ketones. However, this procedure is not as convenient because it requires a hydrogen pressure of 100 to 150 bars (Farlow et al., 1950). Finally, the catalytic substitution of H_2S is the most selective procedure for obtaining primary thiols (mercaptans), but this is not the case for secondary thiols because of the rapid dehydration of alcohols. However, the selectivity for mercaptans is significantly increased when carbonyl compounds are added to alcohols (Barrault et al., 1976, 1977). To explain this effect we proposed (Lucien et al., 1978) a scheme involving two kinds of reactions, namely thiolation of carbonyl compounds into thiocarbonyl compounds by H_2S and reduction of thiocarbonyl compounds by hydrogen transfer from alcohols.